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TECHNICAL MANUSCRIPT 137

ELECTRON MICROSCOPIC AND IMMUNOFLUORESCENT OBSERVATIONS ON YELLOW FEVER INFECTION

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TECHNICAL MANUSCRIPT 137

ELECTRON MICROSCOPIC AND IMMUNOFLUORESCENT OBSERVATIONS ON YELLOW FEVER INFECTION

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ABSTRACT

Immunofluorescence and electron microscopy were used to study changes in liver cells of cynomolgus monkeys and tissue cultures of hamster kidney cells (HKL) infected with the Asibi strain of yellow fever virus. Specific viral antigen was first seen in HKL cells by immunofluorescence at 12 to 15 hours. The amount of antigen increased gradually through 24 hours. Membrane-limited particles containing dense cores were seen at 18 hours in the electron microscope. These particles measured 42 ± 2 millimicrons in diameter. Ultrastructural changes in the HKL cells were limited to a fine vesicular alteration in the endoplasmic reticulum and the appearance of virus particles within vacuoles.

The liver cells of cynomolgus monkeys inoculated with yellow fever virus showed a progressive loss of glycogen and a reorganization of the ribosomes into polyribosomes. Characteristic virus particles were not seen and the immunofluorescent observations corroborated the paucity of parenchymal cells containing mature virus. It was concluded that polyribosomes are formed in hepatic parenchymal cells infected with yellow fever virus, that the polyribosomes are related to synthesis of virus, that the infected cycle is predominantly incomplete, and that the number of virus particles formed in the infected liver cell is small and/or they are rapidly released from the cell.

I. INTRODUCTION

Yellow fever virus infects and kills hepatic parenchymal cells in a variety of mammalian species, i.e. mouse, monkey and man.[1] It also infects and replicates in certain cells in tissue culture. Bearcroft has made extensive observations on the optical and electron microscopic changes in the livers of monkeys infected with the Asibi strain of yellow fever virus.[2-6] His conclusions differ from those of Bergold and Weibel[7] and Bayer and Neilson[8] regarding the morphology of the virus, and his interpretation of the ultrastructural changes in the liver cells are at variance with ours. Some of these differences may be due to the viral strains used and variability of virus-cell reaction from one system to another and in the same system under different conditions.

Appropriately controlled immunofluorescence is among the more specific methods for the identification of viruses. It is particularly useful in the absence of a biological test system or the applicability thereof, and can confirm or contradict the conclusions reached by interpretation of changes in fine structure alone. Therefore, these two techniques, immunofluorescence and electron microscopy, were applied in a study of the changes in liver cells of monkeys and tissue cultures of hamster kidney cells (HKL) infected with yellow fever virus.*

II. MATERIALS AND METHODS

A. VIRUS

The Asibi strain of yellow fever virus was used. It was collected in the serum from infected monkeys at the peak of their febrile response, frozen, and stored at -20°C. The concentration of virus in the serum was 10^9 $MICLD_{50}$ per milliliter.

B. ASSAY

Swiss mice weighing 10 to 14 grams were inoculated intracerebrally with 0.03 milliliter of serial dilutions of blood or homogenized liver. Mouse intracerebral median lethal doses ($MICLD_{50}$) were calculated by the method of Reed and Muench.[9]

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* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

C. ANIMALS

Four non-immune cynomolgus monkeys (Macaca irus) were inoculated intraperitoneally with 1.5×10^3 $MICLD_{50}$ of virus. One monkey was anesthetized and killed with Pentobarbital® (Abbott) at each of the following times after inoculation: 24, 48, 72, and 96 hours.

D. TISSUE CULTURE

A line of hamster kidney cells (HKL) grown in Medium 199 supplemented with 10 per cent calf serum, vitamins, and glutamine at a temperature of 35.5°C was used. Leighton tubes, seeded with 300,000 cells, were incubated for 48 hours before inoculation with virus. Each culture was washed twice with phosphate-buffered saline (PBS), covered with 0.1 milliliter of Medium 199 containing 1×10^7 $MICLD_{50}$ of virus, and incubated for one hour. The cells were then washed four times with PBS, and fresh growth medium was added. Pairs of tubes were harvested every three hours for the first day and then at 36 and 48 hours after infection.

E. IMMUNOFLUORESCENCE

A fluorescein isothiocyanate conjugate was prepared from the globulin fraction of serum from monkeys that had survived infection with the Asibi strain after immunization with the 17-D strain of yellow fever virus. Frozen sections of unfixed liver and coverslip cultures of HKL cells were immersed in absolute alcohol at -70°C before staining. Paired controls in which unconjugated immune sera were used to block the reactivity of the subsequently applied conjugate globulin were used to demonstrate the specificity of the reaction.

F. ELECTRON MICROSCOPY

The HKL cells were washed with PBS, scraped from the wall of the Leighton tube, and lightly centrifuged to form pellets. These pellets and small pieces of liver ($1mm^3$) were fixed in one per cent osmium tetroxide in modified* White's saline, embedded in Epon 812, sectioned on a Servall MT2 ultramicrotome, stained with uranyl acetate, and viewed in an RCA EMU 3F microscope. Plates were taken at magnifications of 3,000 to 20,000 and enlarged photographically as desired.

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* According to method of W. C. Bauer, Personal communication.

G. LIGHT MICROSCOPY

Portions of liver and coverslip cultures of HKL cells were fixed in ten per cent buffered formalin and Carnoys (6:3:1) fixative, stained with hematoxylin and eosin, the periodic acid-Schiff reaction (PAS) and acridine orange G diluted 1:1000 in citrate buffer at pH 3.5.

III. RESULTS

A. HKL CELLS

1. Light Microscopy

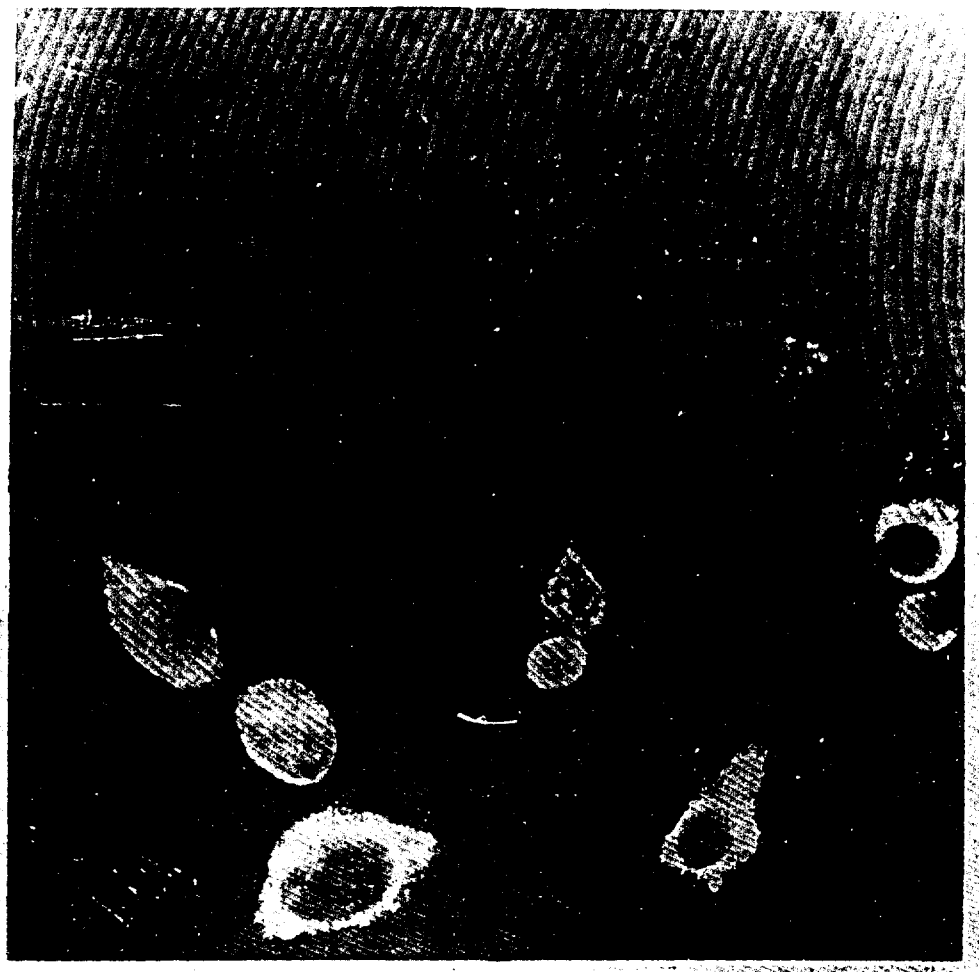
Beginning about 24 hours after infection, subtle changes consisting of rounding up and cytoplasmic vacuolization were noted. Acridine orange staining did not show changes in the amount or distribution of cytoplasmic ribonucleic acid (RNA) or a change in nucleolar size.

2. Immunofluorescence

Specific antigen of yellow fever virus was first detected in the cytoplasm 12 to 15 hours after inoculation. These foci gradually increased so that by 24 to 36 hours the entire cytoplasm of infected cells fluoresced. Figures 1 thru 4 are photomicrographs of immunofluorescent staining of yellow fever virus in a line of hamster kidney cells (HKL). No intranuclear fluorescence was noted at any time. On the basis of this technique it is estimated that one of 10 or 15 cells was infected during the first 24 hours.

3. Electron Microscopy

After 18 hours small numbers of membrane-limited particles containing dense cores were found (Figure 5). The particles measured 42 ± 2 millimicrons in diameter. They were seen on occasion in close association with the endoplasmic reticulum, but when complete (i.e. possessing both dense core and limiting membrane) were within vacuoles and not in the intermembranous compartments of the cytoplasm (Figure 5). No distinct reorganization of the ribosomes was noted. A fine vesicular change occurred in the endoplasmic reticulum. Nuclear and nucleolar changes were indefinite.



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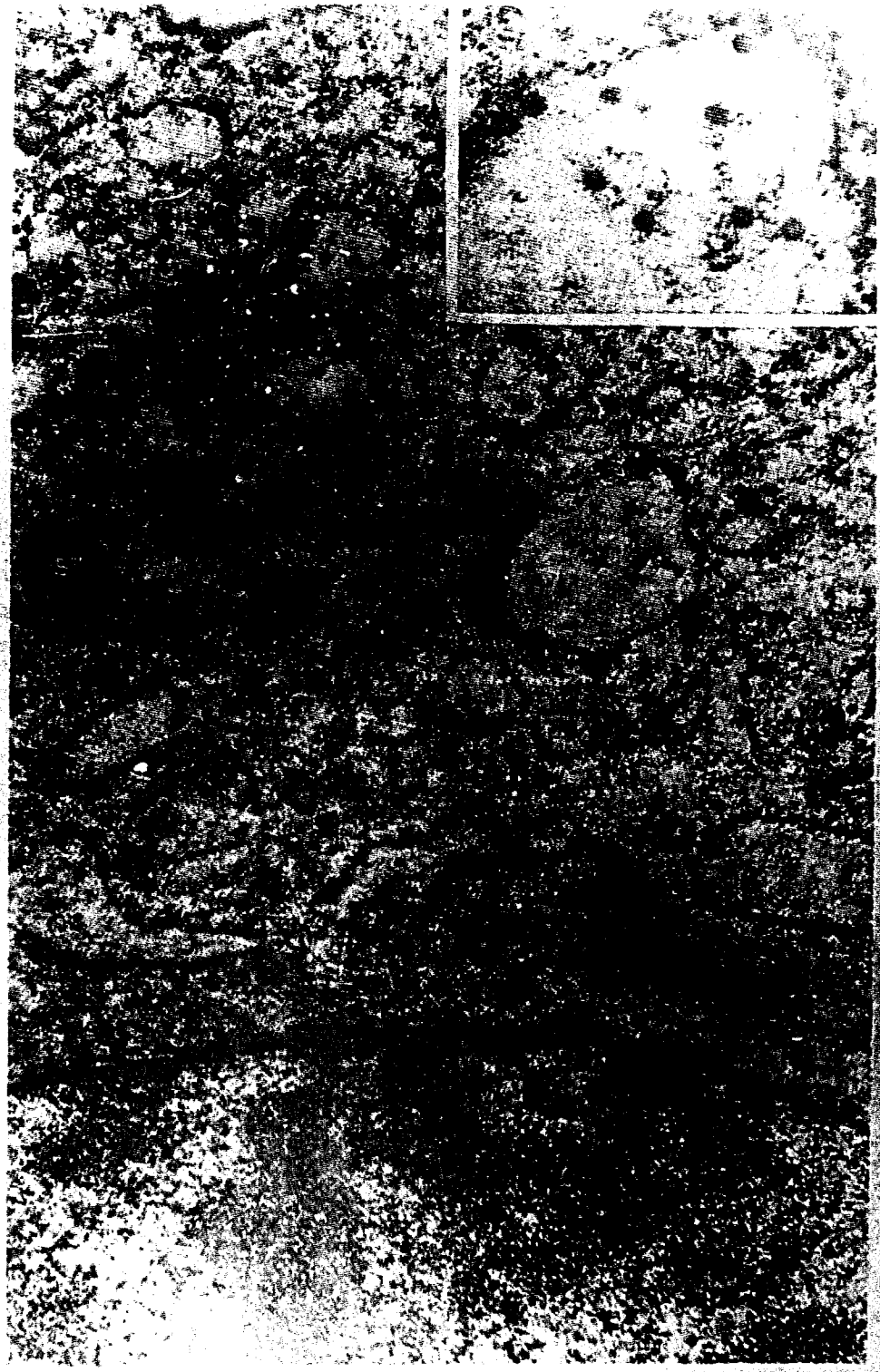

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B. MONKEY LIVERS

1. Light Microscopy

The amount of glycogen in the parenchymal cells was distinctly diminished by 48 hours and none could be detected by PAS stains at 96 hours. By 72 and 96 hours an occasional necrotic Kuppfer cell and a few foci of acute inflammatory reaction in the liver cell cords were apparent. As the glycogen disappeared the cytoplasm became more uniformly basophilic. No frank increment in cytoplasmic RNA was apparent. Nucleolar and nuclear changes were not detected.

2. Electron Microscopy

The changes noted above, the disappearance of glycogen and the redistribution of ribosomes, were corroborated in electron micrographs. In the liver cells of uninfected monkeys and through the first 24 hours after inoculation the ribosomes were associated almost exclusively with membranes forming ergastoplasm (Figure 6). Thereafter this association becomes less exclusive and by 72 to 96 hours ergastoplasm was seldom seen (Figures 7, 8, and 9). In a few liver cells at 48 hours focal aggregates of particles, having the characteristics of ribosomes, were noted. These aggregates, average 100 millimicrons in diameter, and are composed of clusters of 20-millimicron particles. They increased in number and by 96 hours filled the intermembranous areas of the cytoplasm of all the liver cells. Associated with these aggregates of ribosomes was an alteration of the endoplasmic reticulum into numerous small vesicles (Figure 9). No characteristic virus particles were found in the parenchymal cells, the space of Disse, the Kuppfer cells, or the biliary epithelium. Degenerative changes such as swelling and fragmentation of mitochondria, nuclear vacuolization, rupture of cell membranes, and increase in dense bodies were not seen through 96 hours.

3. Immunofluorescence

A few foci of specific fluorescence were found in the livers at 48 and 72 hours. The cells demonstrating this reaction were probably parenchymal. At 96 hours no reaction was elicited in any cells.

4. Virus Assay

The titers of virus in the blood and liver of the four monkeys are shown in Table I.

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Figure 6. Hepatic Parenchymal Cell from a Monkey 24 Hours after Inoculation. Large areas of the cell are filled with glycogen, which has a faintly granular grey appearance in this uranyl-acetate-stained section. The ribosomes are membrane-associated. About the bile caniculus, at the bottom, two zones filled with smooth membranes are seen. A portion of the space of Disse and a sinusoid are seen at the upper left. The nucleus and nucleolus are not altered. 7500X

Figure 7. At 48 Hours the Smooth-Surfaced [illegible] and the Amount of Glycogen [illegible] varying size and electron [illegible] caniculus and Golgi apparatus [illegible] of liver cells. Several [illegible] are shown. The [illegible]

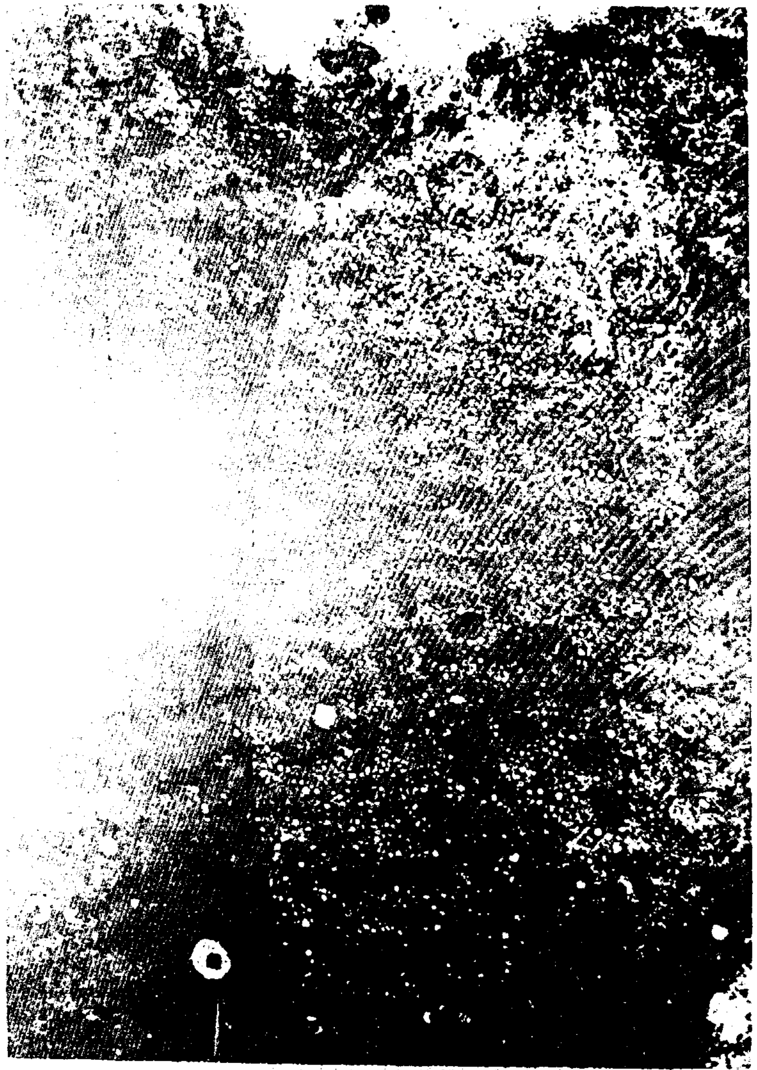

Figure 8. By 96 Hours the Entire Cytoplasm of the Liver Cells is Filled by Small Aggregates and Fine Vesicles. No glycogen or ergastoplasm is seen. Portions of four contiguous cells show comparable filling of their cytoplasm by these aggregates. The mitochondria are intact and dense bodies are not increased. 6000X

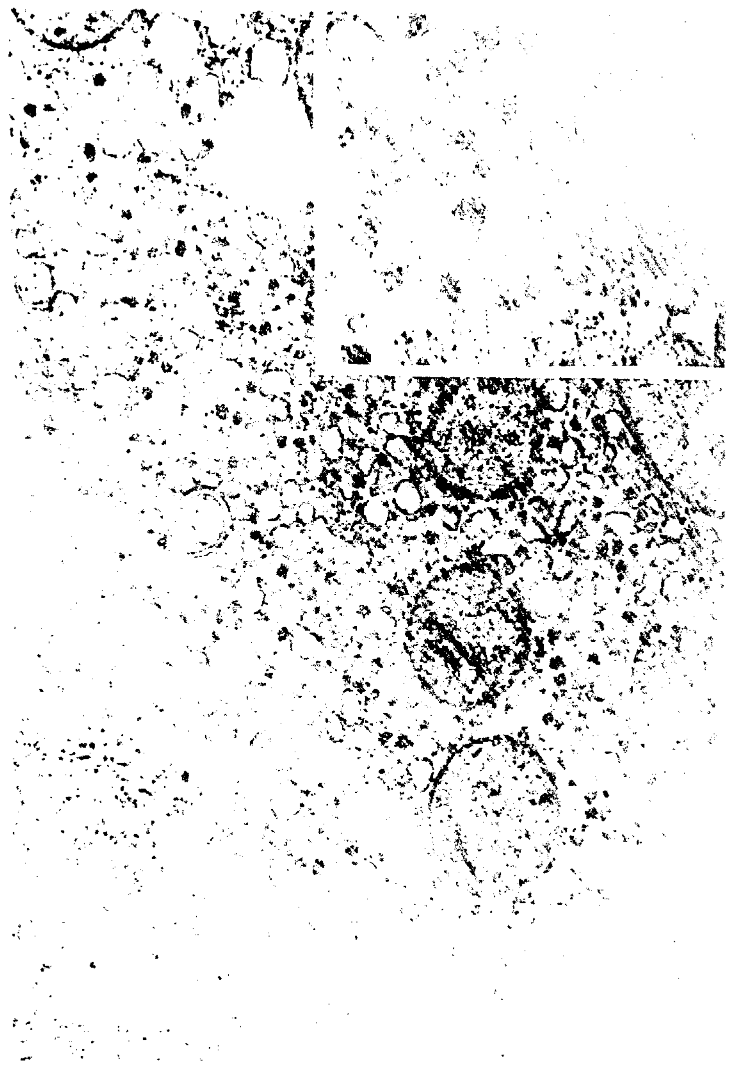

Figure 9. In a Parenchymal Cell at [illegible] Form in the Cytoplasm [illegible] They are not seen within [illegible] reticulum. Free ribosomes [illegible] scattered along the top [illegible] insert shows the [illegible] 24,750X. Insert, [illegible]

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TABLE I. CONCENTRATIONS OF YELLOW FEVER VIRUS IN LIVER AND BLOOD OF CYNOMOLGUS MONKEYS INOCULATED INTRAPERITONEALLY WITH 1.5×10^3 $MICLD_{50}$'s OF VIRUS

Time Post Inoculation, hr	Sample	Log Virus Concentration, $MICLD_{50}$ per ml Log 10
24	Liver	4.5
	Blood	3.3
48	Liver	6.4
	Blood	3.5
72	Liver	6.7
	Blood	4.1
96	Liver	3.2
	Blood	2.8

IV. DISCUSSION

Whether the diminution of glycogen in the liver cells was directly related to virus replication is uncertain. Anorexia might have produced this change. However, in certain virus-cell systems, glycolysis is enhanced.

The conglomerates of ribosomes in liver cells have been thought to be virus by Bearcroft[4] in his studies of yellow fever in rhesus monkeys. Bearcroft,[10] Kikkawa and Gueft,[11] and Taylor et al[12] have made the same assumption from their observations of liver cells from men with infectious hepatitis. The absence of virus antigen in these aggregates and the differences in size and form of these aggregates and the structure of the virus of yellow fever as shown by Bergold and Weibel, Bayer and Neilson, and in the HKL cells in this report cast doubt on such an assumption. All of these workers have shown that the individual component of the "virus" measures about 20 millimicrons, the size of ribosomes. The variety of sizes for the "viruses" quoted are then dependent on the number and packing in the aggregate.

It appears, in light of the recent observations of Warner et al,[13] that such aggregates of ribosomes are in fact polyribosomes. These workers have shown that protein synthesis —hemoglobin in reticulocytes[14,15] and poliovirus in Hela cells[16] —is associated with polyribosomes and the number

of ribosomes in the polyribosomes is proportional to the size of the molecule being synthesized. In the HeLa cells infected with poliovirus a distinct difference in the spectrum of sizes of polyribosomes is found between infected and uninfected cells.

If these structures in the liver cells infected with yellow fever virus are polyribosomes and if they are producing viral protein, one might expect to find complete virus. None was observed in the electron micrographs.*

The immunofluorescent observations document the paucity of parenchymal cells containing mature virus in far larger samples than that used in electron microscopy. Two explanations for these seemingly paradoxical findings are tenable. On the one hand, the cycle of yellow fever infection in liver cells may be short and the relatively small numbers of mature virus particles released rapidly. On the other hand, the cycle may be predominantly incomplete. Further study will be necessary to determine which of the alternatives is correct.

Upon the basis of the data presented here, it seems consistent to conclude that the ultrastructural changes seen in hepatic parenchymal cells infected with yellow fever virus are related to the formation of polyribosomes; that these are concerned with the synthesis of virus; that the infective cycle may be generally incomplete; and that the number of virus particles formed in the infected liver cell is small, and/or that the viruses are rapidly released and thus not readily found in thin sections. The determination of the mechanisms whereby the liver cells are killed awaits the application of the available analytic techniques.

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* The probability of finding virus in thin sections may be estimated within a reasonable range. Assume that the total number of complete viruses made in a 5-kilogram monkey is 10^{12}. This estimate is based on a peak viremia of 10^7 $MICLD_{50}$ per milliliter, a blood volume of 250 milliliters, a similar titer of virus in a 250-gram liver, and an assumption that one $MICLD_{50}$ requires 100 infective particles. Hence, 10^7 $MICLD_{50}$ x 10^2 = 10^9 virus particles; 500 x 10^9 = 5 x 10^{11} or 1 x 10^{12}. Assume 10^{13} liver cells in a 250-gram liver. Then if all the virus were in the liver cells, which it is not, there would be 10 liver cells for every virus particle. One may cut one hundred thin sections from a single liver cell; this estimate may be modified up or down. Assume that the total virus synthesized is 10^{15} particles and that at 96 hours one half is within one one-thousandth of the liver cells ($10^{7.5}$ particles, 10^{13} - 10^3 = 10^{10} liver cells). The odds of finding virus in sections are now even less, there being 250 cells per virus particle.

V. SUMMARY

When hamster kidney cells in tissue culture are inoculated with the Asibi strain of yellow fever virus, specific viral antigen appears in the cytoplasm by 12 to 15 hours and gradually increases in amount. Ultrastructural examination of these cells shows the appearance of characteristic virus particles that measure 42 ± 2 millimicrons. The liver cells of cynomolgus monkeys inoculated with yellow fever virus show a progressive loss of glycogen and a reorganization of the ribosomes into clusters. Viral antigen is not demonstrable in these cells and no structure characteristic of a virus particle is found in them. It is hypothesized that these clusters of 20-millimicron particles having the appearance of ribosomes are polyribosomes and not virus.

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